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14. ABSTRACT
Background: The majority of patients with advanced breast cancer develop bone metastases, which are incurable. Recently, tumor-secreted factors have shown promise as targets for the treatment of bone metastasis. Adrenomedullin (AM) is a breast cancer-secreted peptide that is pro-proliferative, anti-apoptotic, pro-angiogenic, and stimulates new bone formation. AM overexpression increases bone metastases while AM knockdown decreases bone metastases in mouse models of prostate and lung cancer respectively. Objective/Hypothesis: The objective of this project is to validate AM as an important target for the treatment of breast cancer bone metastasis. I hypothesize that AM expression increases bone metastases and resistance to chemotherapy. Specific Aims: (1) To determine if AM expression by breast cancer cells increases bone lesion formation in bone metastasis mouse models. (2) To determine the role of AM in breast cancer cells. Key Research Accomplishments: (1) MDA-MB-231 clones that overexpress AM were produced. (2) Stable AM shRNA knockdown MDA-MB-231 breast cancer cell clones were produced. (3) Decreasing AM in breast cancer cells increased bone lesion formation but decreased mammary fat pad tumor-take and growth in mice. Relevance: Currently no treatments improve overall survival for breast cancer bone metastasis patients. Inhibitors of certain tumor-secreted factors have decreased bone metastases in mice. My results indicate that inhibitors of the tumor-secreted factor AM would not be a good treatment for breast cancer bone metastases.
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bone metastasis, adrenomedullin, tumor-secreted factors

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Introduction:

The majority of patients who develop advanced breast cancer develop bone metastases, which are incurable (Siclari et al, 2006). Bone metastasis patients suffer from extreme bone pain, skeletal fractures, nerve compression, and hypercalcemia (Siclari et al, 2006). Current treatment, the use of antiresorptive bisphosphonates, reduces bone pain and skeletal fractures but does not improve overall survival (Siclari et al, 2006). The incurability of the disease is produced by a 'vicious cycle' that develops between the tumor cell and the bone microenvironment (Siclari et al, 2006). Once the tumor cell has entered bone, the tumor cell secretes factors that act on bone cells and other surrounding cells, causing them in turn, to secrete factors back onto the tumor cells (Siclari et al, 2006). Inhibiting tumor-secreted factors has led to decreased bone metastases in mice (Siclari et al, 2006). Currently, inhibitors of two tumor-secreted factors are under clinical trials for bone metastasis treatment (Siclari et al, 2006). My project is studying the tumor-secreted factor adrenomedullin (AM) and its role in breast cancer bone metastasis. AM is a 52 amino acid peptide that is pro-proproliferative, anti-apoptotic, pro-angiogenic, and induces new bone formation (Zudaire et al, 2003, Cornish et al, 2003). Overexpressing AM increased bone metastasis formation while decreasing AM decreased bone metastasis formation in prostate and lung cancer mouse models respectively [unpublished data]. My hypothesis is that AM is a causal factor in breast cancer bone metastasis that increases lesion formation and chemoresistance. To test this hypothesis, I plan to look at the effect of stable overexpression and knockdown of AM in breast cancer cells on bone lesion formation, cell proliferation, chemoresistance, and cell migration and invasion.

Body:

Task 1: Training in how to produce AM-overexpressing stable clones. Create and test stable AM-overexpressing breast cancer cell lines for mouse study.

Since my AM knockdown bone metastasis experiment indicated that the role of AM is cell-type specific, I decided to perform my AM overexpression also using MDA-MB-231 cells (see task 6). The original plan was to use MCF-7 cells but MCF-7 cells normally behave differently in the bone than MDA-MB-231 cells, which may have added a further confounding factor. Therefore, I chose to focus specifically on the role of AM in an osteolytic breast cancer cell line (MDA-MB-231).

A hAM and an emerald Green Fluorescent Protein (GFP control) expression vector were stably transfected into MDA-MB-231 breast cancer cells to produce stable pools with about a 2 fold increase in AM mRNA and green fluorescence. Single cell AM-overexpression and GFP-control clones were isolated using a limited dilution technique (Figures 1 & 2). Eight AM-overexpressing clones with 6 fold or greater AM overexpression along with 8 GFP-overexpressing clones were selected to test for stability. Clonal stability is determined by culturing the cells without antibiotic for sixty days. Five out of the eight GFP clones maintained detectable levels of fluorescence after 30 days of culture without antibiotic selection as observed by a fluorescent microscope (Figure 2). Six out of the eight AM-overexpressing clones maintained increased AM mRNA levels compared to the GFP control and parental cells after 30 days of culture without antibiotic treatment determined by RealTime PCR (Figure 1).

After completion of the remainder of the 60 day stability test, two GFP control and two AM overexpressing clones will be placed into the mouse model and I will determine if increasing AM increases bone lesion formation and chemoresistance (Tasks 2, 3, and 7). Experiment is tentatively planned for July.

Task 4: Training in the use of siRNA to produce stable AM-knockdown clones. Create and test stable AM knockdown MDA-MB-231 cells for mouse study using siRNA.

MDA-MB-231 AM knockdown clones were produced that stably maintained about a 90% AM knockdown for sixty days without antibiotic selection (Figure 3).

Task 5: Create mouse models of breast cancer bone metastasis using stable AM knockdown and control MDA-MB-231 breast cancer cells

Two AM knockdown and two control MDA-MB-231 clones were injected into the left cardiac ventricle of female nude mice to produce a mouse model of bone metastasis. Twelve mice were injected per group. Bone lesion formation was monitored by x-ray and bones were collected at death for histologic analysis.

The same two AM knockdown and control MDA-MB-231 clones were also injected into the mammary fat pad of female nude mice. Ten mice were injected per group and two mammary fat pads were injected per mouse. Tumor-take and size was monitored.

Task 6: Analyze data from MDA-MB-231 AM knockdown mouse bone metastasis experiment using x-rays and bone histomorphometry

X-ray analysis revealed that AM knockdown produced the opposite effect of what I had hypothesized to happen in the breast cancer bone metastasis model (Figure 4). AM

knockdown clones caused greater osteolytic lesion area in mice (17.9 vs. 4.8 mm2 at 5wks; $p<0.0001$) compared to control clones as measured by x-ray analysis. Further analysis of this experiment by bone histomorphometry is currently underway. Bone histomorphometry will reveal differences in total bone area, tumor area, and osteoclast numbers.
AM knockdown had the opposite effect in bone than it did in the mammary fat pad (Figure 5). AM knockdown clones exhibited decreased tumor take (2.5% vs 64.3%; $p<0.0001$) and growth (0.5mm3 vs. 126.4mm3 on day 26) within the mammary fat pad compared to the scrambled control clones.
This data shows that AM is a complex peptide. Both the cell type and environment affect AM's role in cancer. For this reason, I chose to use MDA-MB-231 cells again in our over-expression experiment to further support our data. My new hypothesis is that AM is a factor that leads to decreased osteolytic lesion formation but increased mammary fat pad growth.

Task 8: Test the gene changes induced by AM treatment of breast cancer cells using Q-PCR.
In my last report, I noted gene differences between my AM knockdown and control clones. AM knockdown clones had a statistically significant decrease in both IL-11 and ET-1 mRNAs compared to the control clones. I looked to see if AM overexpression also altered the levels of IL-11 and ET-1 but found no difference in IL-11 and ET-1 mRNA between my AM-overexpressing clones and my control GFP clones (Figure 6). This data suggest that the decrease in IL-11 and ET-1 in my AM knockdown cells may just have been due to clonal variation. My additional data does not support a role of AM in regulating IL-11 and ET-1.

Task 10: Write papers and defend thesis project
I have published a chapter in a book entitled Breast cancer secreted factors alter the bone microenvironment: Potential new targets for bone metastasis treatment in Metastasis of Breast Cancer.

Summary of Specific Aims and Completed Tasks:

Specific Aim 1: **To determine the effects of tumor AM on bone**

Specific Aim 1.1: To determine the effects of increasing tumor-expressed AM in MCF7 cells on bone metastasis

Task 1: (Specific Aim 1.1) Training in how to produce AM-overexpressing stable clones.
Create and test stable AM-overexpressing breast cancer cells for mouse study.
Task complete

Task 2: (Specific Aim 1.1) Training in how to produce mouse models of bone metastasis and training in how to analyze mouse model results.

Create mouse models of breast cancer bone metastasis using stable AM-overexpressing breast cancer cells.
Task planned for summer 2008.

Task 3: (Specific Aim 1.1) Analyze data from mouse experiment for Specific Aim 1.1 using x-rays, bone histology, and histomorphometry.
Completion of this task will reveal whether overexpression of AM in MCF7 cells increases bone lesion formation in mice.
Task will be complete in the next couple of months.

Specific Aim 1.2: To determine the effects of decreasing tumor expression of AM in MDA-MB-231 cells on bone metastasis

Task 4: (Specific Aim 1.2) Training in the use of siRNA to produce stable AM-knockdown clones.
Create and test stable AM knockdown MDA-MB-231 cells for mouse study using siRNA.
Task complete

Task 5: (Specific Aim 1.2) Create mouse models of breast cancer bone metastasis using stable AM knockdown MDA-MB-231 clones and control MDA-MB-231 breast cancer cells.
Task complete

Task 6: (Specific Aim 1.2) Analyze data from mouse experiment for Specific Aim 1.2 using x-rays, bone histology, and histomorphometry.
Completion of this task will reveal whether decreasing the expression of AM decreases bone lesion formation in mice.
Task underway.

Specific Aim 2: **To determine the role of AM in Breast Cancer cells**
Specific Aim 2.1: To determine if AM increases resistance of tumor cells to chemotherapy

Task 7: (Specific Aim 2.1) Test the effects of AM-overexpression in breast cancer cells on sensitivity to taxol using MTT assays.
Completion of this task will reveal whether AM promotes chemoresistance to taxol.
Task will be completed in June.

Specific Aim 2.2: To determine if genes regulated by AM in bone cells are also regulated similarly in breast cancer cells

Task 8: (Specific Aim 2.2) Test the gene changes induced by AM treatment of breast cancer cells using Q-PCR
Completion of this task will reveal the mechanism of AM action in breast cancer. This task may also reveal additional targets for the treatment of breast cancer.

Task complete.

Specific Aim 2.3: To determine the regulation of AM expression in breast cancer cells by BRCA1 and whether this regulation is via Rho signaling and the metastasis suppressor Rho GDI2, a negative regulator of Rho kinase signaling

Task 9: (Specific Aim 2.3) Test role of Brca1 and Rho signaling in the regulation of AM expression in breast cancer cells using siRNA, transient transfections, and Q-PCR. Completion of this task will reveal whether or not Rho signaling regulates AM expression in Breast Cancer cells and will indicate a possible mechanism to decrease AM levels in Breast Cancer.
Task complete. No evidence that RhoGDI2 regulates AM. Abandon task.

Task 10: Write papers and defend thesis project
Completion of this task will result in the publication of at least one paper in a well-respected journal and the achievement of a Ph.D in biochemistry and molecular genetics.
Task in progress
Book chapter was published in 2007.

Key Research Accomplishments:

- Determined that AM knockdown in breast cancer cells increases bone lesion formation in mice.
- Determined that AM knockdown in breast cancer cells decreases tumor take and growth in the mammary fat pad of mice.
- Produced AM overexpressing breast cancer cells that will be used to determine the effects of increasing AM on breast cancer bone metastasis.

Reportable Outcomes:
6/2007: Book chapter published in the book entitled: Metasis of Breast Cancer v.11
Title: Breast cancer secreted factors alter the bone microenvironment: Potential new targets for bone metastasis treatment
9/2007: Abstract/Poster Presentation at the annual meeting of the American Society of Bone and Mineral Research
Title: Development of Small Molecule Adrenomedullin Antagonists for Treatment of Bone Metastases
10/2007: Abstract/Poster Presentation at the Paget's Foundation Skeletal Complications of Malignancy Meeting
Title: The Role of Adrenomedullin in Cancer, Bone, and Metastasis
12/2007: Abstract/Oral Presentation at the Virginia Branch of the American Cancer Society Meeting
Title: The Role of Adrenomedullin in Breast Cancer Bone Metastasis

Conclusion:

Since the current treatment for breast cancer bone metastases does not cure the disease, new treatments need to be developed. I hypothesized that the breast cancer-secreted peptide adrenomedullin (AM) was a causal factor in breast cancer bone metastasis and inhibiting AM may decrease breast cancer bone metastases. However, my AM knockdown experiment demonstrated the opposite of what I had hypothesized. AM knockdown increased breast cancer osteolytic lesion formation. Though, interestingly, AM knockdown had the opposite effect in the mammary fat pad. AM knockdown decreased breast tumor take and growth within the mammary fat pad. This data differs from our previous data in which AM overexpression in PC3 prostate cancer cells increased bone metastases and knockdown in A549 lung cancer cells decreased bone metastases. However, our PC3 data supports the idea that the microenvironments affects AM's role in cancer. While AM overexpression in PC3 cells increased bone metastases, it decreased primary tumor growth. Therefore, the role of AM in cancer and bone metastasis appears to be very complex and influenced by both the environment and cell type. For this reason, we chose to perform the AM overexpression experiment using the same cell type as the AM knockdown experiment. We now hypothesize that AM overexpression will lead to decreased osteolytic lesion formation but increased mammary fat pad growth.

Inhibitors of AM are currently being developed in preclinical models to treat breast cancer and my data suggests that these inhibitors should be used with caution. While inhibiting AM may lead to decreased primary breast tumor growth, AM inhibitors may cause increased breast cancer bone metastases.

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Appendices:

Abstract: Development of Small Molecule Adrenomedullin Antagonists for Treatment of Bone Metastases

Siclari VA, Mohammad KS, Martinez A, Gineste C, Geysen HM, Guise TA, Chirgwin JM

Adrenomedullin (AM) is a 52 amino acid peptide of the calcitonin/CGRP gene family. AM is secreted by cancers such as breast, lung, and prostate, where it can stimulate angiogenesis and autocrine signaling. AM also dose-dependently stimulates new bone formation at picomolar concentrations, by binding to the calcitonin-receptor-like receptor plus RAMP2 or 3 and stimulating adenyl cyclase. However, the mechanisms by which AM induces new bone formation are incompletely understood. AM treatment stimulates osteoblast proliferation but does not induce bone matrix protein (bone sialoprotein, type I collagen, osteocalcin, and osteopontin) mRNA expression. AM stimulates IL-6 mRNA expression by primary osteoblasts. We previously reported increased and decreased bone metastases due to AM overexpression and siRNA knockdown respectively in prostate and lung cancer models. These observations identify AM as a significant target for therapeutic intervention in bone metastasis.

Small molecules have been identified that function as agonists or antagonists of the action of AM to increase cAMP. They bind to the AM ligand rather than the receptor. One of these antagonists, NCI 16311, binds with Kd = 8nM to AM without altering receptor binding affinity. 100nM 16311 was added to cultures of neonatal mouse calvariae. The increases in new bone and osteoblast number caused by 1nM AM were completely blocked by 16311, without cellular toxicity or blockade of new bone formation due to IGF1 receptor activation. However, 20nmol/kg 16311 iv dramatically increased blood pressure in rats. Two additional antagonists were tested in the same assays. NCI 28086 was ineffective, but NCI 37133 was as effective as NCI 16311 at blocking AM-induced new bone formation, while it did not increase blood pressure in rats. Thus it may be possible to develop effective bone-selective antagonists of tumor-secreted AM. NCI compounds 16311 and 37133 are aromatic carboxylic acid derivatives that act extracellularly. Further development of these compounds into higher affinity, second-generation derivatives should be possible. Small molecule AM antagonists may lead to improved treatment for bone metastasis.

Abstract: The Role of Adrenomedullin in Cancer, Bone, and Metastasis

Siclari VA, Mohammad KS, Martinez A, Gineste C, Geysen HM, Guise TA, Chirgwin JM

Adrenomedullin (AM) is a 52 amino acid peptide of the calcitonin/CGRP gene family. AM is secreted by cancers such as breast, lung, and prostate, where it can stimulate angiogenesis and autocrine signaling. AM also modulates the actions of microtubules and preliminary results indicate that AM may interfere with taxol (a microtubule-binding chemotherapeutic) treatment of breast cancer cells. AM dose-dependently stimulates new bone formation at picomolar concentrations, by binding to the calcitonin-receptor-like receptor plus RAMP2 or 3 and stimulating adenyl cyclase. However, the mechanisms by which AM induces new bone formation are incompletely understood. AM treatment stimulates osteoblast proliferation but does not induce bone matrix protein (bone sialoprotein, type I collagen, osteocalcin, and osteopontin) mRNA expression. AM stimulates IL-6 mRNA expression by primary osteoblasts. We previously reported increased and decreased bone metastases due to AM overexpression and siRNA knockdown respectively in prostate and lung cancer models. These observations identify AM as a significant target for therapeutic intervention in bone metastasis.

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Abstract: THE ROLE OF ADRENOMEDULLIN IN BREAST CANCER BONE METASTASIS

VA Siclari*, K Mohammad, M Niewolna, C McKenna, H Walton, L Wessner, TA Guise, JM Chirgwin

The majority of women with advanced breast cancer develop incurable bone metastases, which cause bone pain, skeletal fractures, hypercalcemia, nerve compression, and morbidity. Current antiresorptive treatments do not improve survival. Tumor-secreted factors are promising targets for treatment of bone metastasis. Such factors stimulate bone cells, which in turn secrete factors that stimulate the tumor cells leading to an incurable vicious cycle. Adrenomedullin (AM), a 52 amino acid peptide is commonly secreted by breast cancer cells and stimulates osteoblasts *in vivo* and *in vitro* making it a potential treatment target. AM overexpression in prostate cancer cells increased bone metastases in mice, while siRNA knockdown of AM in lung adenocarcinoma cells decreased bone lesions. We hypothesized that decreasing AM expression in breast cancer cells would decrease bone metastases.

MDA-MB-231 breast cancer cells were transfected with either a shRNA expression vector for AM or a control scrambled shRNA sequence. Single-cell AM knockdown and control shRNA clones were isolated. Knockdown was confirmed using real-time PCR. Clones were cultured for sixty days without antibiotic treatment and AM mRNA was re-analyzed by PCR to confirm clonal stability. Several clones were tested for expression of tumor-secreted factors by PCR. *In vitro* proliferation was measured by MTT assay. Two stable AM shRNA and two negative control shRNA clones were injected into the left cardiac ventricle of nude mice to form a bone metastasis mouse model. Bone lesions were monitored by x-ray. *In vivo* shRNA vector expression was confirmed by bone marrow flushing and PCR.

Stable AM mRNA was knocked down 90% in MDA-MB-231 cells. AM knockdown clones had decreased IL-11 and ET-1 mRNA levels but no change in Cyr61, CTGF, IL-8, PTHrP, and IL-6 mRNA levels compared to scrambled control clones. Decreased IL-11 or ET-1 mRNAs were not rescued by adding exogenous AM, suggesting an intracrine action of AM. No significant cell morphological differences were noted by microscopy. Knockdown clones were more sensitive to growth inhibition by taxol *in vitro* than controls. The knockdown clones caused more total bone lesion area in mice by xray compared to control clones. All clones maintained expression of the shRNA vector *in vivo* as measured by neomycin resistance gene expression.

Decreasing AM promotes bone metastases by osteolytic breast cancer cells. Its role in breast cancer is different than in prostate and lung cancers. Decreasing AM increased bone lesions perhaps by increasing cancer cell proliferation. Bone histomorphometry will assess changes in osteoclast and osteoblast numbers at metastatic sites in response to AM knockdown and a mammary fat pad experiment to assess effects on tumor growth is underway.

Chapter #

BREAST CANCER SECRETED FACTORS ALTER THE BONE MICROENVIRONMENT

Potential New Targets for Bone Metastasis Treatment

Siclari VA, Guise TA, and JM Chirgwin
University of Virginia

Abstract: Bone is the most common site of breast cancer metastasis. Over eighty percent of patients with advanced breast cancer develop bone metastases. Once breast cancer has spread to bone, the cancer is incurable and patients develop mostly osteolytic, but also osteoblastic, or mixed bone lesions and suffer from extreme bone pain, skeletal fractures, hypercalcemia, and nerve compression. Current treatment is the use of antiresorptive bisphosphonates, which reduces bone pain and skeletal fractures but does not improve overall survival. Mouse models of bone metastasis have led to an understanding of the complex interactions that occur within bone that contribute to the incurability of the disease. Once breast cancer cells enter bone, a 'vicious cycle' develops between breast cancer cells and the other cells within bone. Breast cancer cells secrete factors that stimulate bone cells, causing them in turn to secrete factors back onto the cancer cells. Inhibiting the actions of cancer-secreted factors may break this vicious cycle. The list of tumor-secreted factors is long, but they can be divided into three groups: (1) bone-resorbing, (2) bone-forming, and (3) metastasis-opposing factors. These factors may share upstream regulatory pathways. Such central pathways could provide new targets for more effective treatment of bone metastasis. The TGFβ and hypoxia-induced Hif1α pathways are two leading targets for such adjuvant treatments.

Key words: Breast Cancer, Bone Metastasis, Tumor-Secreted Factors

1. INTRODUCTION:

Breast cancer is one of several cancers, including lung and prostate cancer that displays osteotropism or a preferential growth in bone[1]. Bone is

the most common site of breast cancer metastasis, and over eighty percent of patients with advanced breast cancer will develop bone lesions and suffer from skeletal fractures, hypercalcemia, bone pain, or nerve compression[2]. Bone metastases are currently incurable[2]. The approved treatment, antiresorptive bisphosphonates, is only palliative[2]. Median survival from time of diagnosis of bone metastases is about two years[2]. Therefore, new treatments need to be identified to cure this disease. Understanding why breast cancer spreads to bone and aspects of both the breast cancer cell and the bone microenvironment may reveal new targets. This chapter focuses on breast cancer-secreted factors with the goal of identifying molecular targets for improved treatment.

2. THE 'SEED AND SOIL' HYPOTHESIS: AN EXPLANATION FOR THE PREFERENTIAL SPREAD OF CANCER CELLS

The 'Seed and Soil Hypothesis' was proposed by Stephen Paget in 1889 to explain the preferential spread of breast cancer to bone[3, 4]. It states: "when a plant goes to seed, its seeds are carried in all directions; but they can only grow if they fall on congenial soil"[4]. The 'seed' is the breast cancer cell, which can only grow or form metastases in particular, compatible parts of the body or 'soils'[3]. Aspects of both the seed and the soil contribute to the successful formation of a metastasis[3]. Not every seed can grow in every soil[3]. In the case of breast cancer, bone serves as a fertile 'soil' for the breast cancer 'seed' to grow.

3. BONE: A FERTILE SOIL FOR THE BREAST CANCER SEED

The mineralized matrix of the bone is a rich store of growth factors and calcium that are released during bone resorption[5]. The released growth factors contribute to the growth of breast cancer cells in bone[6]. Insulin-like growth factors (IGFs) I and II and transforming growth factor β (TGFβ) are the most abundant growth factors in bone[5]. A role of bone matrix IGF I and II in bone metastasis has not been completely demonstrated. Currently, only TGFβ has been shown to be actively released from the bone matrix by osteoclast resorption[7]. Expression of a dominant negative TGFβ receptor subunit in MDA-MB-231 breast cancer cells blocked responsiveness to

TGFβ and decreased bone metastases in mice[8]. TGFβ inhibitors are effective in preclinical models to block bone metastases[9-12].

Actions of the two main bone cell types are coupled. The bone-forming osteoblast and the bone-resorbing osteoclast maintain bone homeostasis by a process of remodeling[13]. Osteoclasts resorb bone, leaving a pit within which osteoblasts then form new bone[13]. Osteoclast formation is regulated by cells of the osteoblast lineage that express macrophage-colony stimulating factor (M-CSF) and receptor activator of NFkappaB ligand (RANKL)[14]. M-CSF induces monocyte/macrophage cell precursors to express the receptor activator of NFkappaB (RANK)[14]. Binding of RANKL to RANK stimulates the differentiation of the precursor cells into osteoclasts and increases osteoclast activation and survival[14]. Imbalances in the activities of osteoblasts and osteoclasts can lead to increased bone loss or bone formation[14]. Breast cancer cells in bone cause such imbalances, producing predominantly osteolytic (bone destructive), but also osteoblastic (bone forming) and mixed bone lesions[14].

4. BONE METASTASIS MOUSE MODELS AND THE VICIOUS CYCLE

Only the murine mammary carcinoma 4T1 model spontaneously forms metastases to the bone, but it also spreads to the liver, lungs, and brain[15]. Standard bone metastasis models are produced by injecting cancer cells into the left cardiac ventricle of immunocompromised mice[6]. Within this model, MDA-MB-231 human breast cancer cells produce osteolytic bone lesions within a month after tumor cell inoculation[6]. MDA-MB-435s and BT549 breast cancer cell lines also produce osteolytic lesions[6]. Other breast cancer cell lines produce osteoblastic (T47D, MCF-7, ZR75.1) or mixed (BT483) bone lesions within this model[6, 16]. These bone metastasis mouse models have led to an understanding of the complex interactions that develop between breast cancer cells and the bone microenvironment that lead to lesion formation and the incurability of the disease[6], although they lack important regulators of cancer progression such as T lymphocytes. Data from these models provide evidence that a ‘vicious cycle’ develops between breast cancer cells and the other cells within bone[6]. Once breast cancer cells have entered bone, they secrete various factors that act on bone cells and other cells within the bone, causing them to secrete factors back onto the breast cancer cells, driving a ‘vicious cycle’ that renders the disease

incurable[6]. Inhibiting the secreted factors may interfere with the vicious cycle and lead to a cure for breast cancer bone metastasis[6].

5. TUMOR-SECRETED FACTORS:

Breast cancer cells secrete many factors that in combination contribute to bone metastases[17]. They can be broken down into two groups: (1) bone-resorbing and (2) bone-forming factors[14]. Osteolytic breast cancer-secreted factors include: PTHrP, IL-11, IL-6, VEGF, IL-8, CSFs, EGF, oxygen-derived free radicals, PDGF, prostaglandins, PTH, TNFs, TGFs, and IL-1[14, 18]. Potential osteoblastic factors include: ET-1, stanniocalcins, AM, many of the six CCN proteins, BMPs, PTHrP fragments generated by PSA proteolysis, BDGF, FGFs, IGFs, PDGF, prostaglandins, TGFβ, TNFs, and urokinase (uPA)[14, 18, 19]. A third group of breast cancer-secreted factors may oppose the development of bone metastases[14]. These factors are often downregulated in breast cancer cells or upregulated as an anti-tumor host response. They include IL-18, IL-4, IL-12, OPG, BMP antagonists such as noggin, and Wnt signaling antagonists (DKKs and soluble frizzled related proteins)[14].

The large list of breast cancer-secreted factors makes the task of identifying the best targets daunting. Some of these factors play roles in other diseases, for which drugs/inhibitors have already been developed and tested. Understanding the role of these particular factors in breast cancer bone metastasis provides the opportunity to translate existing drugs into the clinic for improved treatment of metastases. The rest of this chapter focuses on tumor-secreted factors with established roles in breast cancer bone metastasis; potential new treatment targets will be highlighted.

5.1 Bone-Resorbing Breast Cancer-Secreted Factors:

Breast cancer-secreted factors induce bone resorption by both indirect and direct actions on the osteoclast. Parathyroid hormone-related protein (PTHrP) is the most studied breast cancer-secreted factor. It indirectly activates osteoclastic bone resorption by stimulating osteoblasts and stromal cells to express RANKL, which in turn activates osteoclasts[20]. PTHrP was first identified as a causal factor in humoral hypercalcemia of malignancy and was later shown to be a major factor in promoting osteolytic metastases[14]. Breast cancer cells that have metastasized to bone express higher PTHrP mRNA levels than in soft tissue sites[21, 22]. Inhibiting PTHrP

with neutralizing antibodies decreased osteolytic bone metastases formed by MDA-MB-231 breast cancer cells in mice[23]. A humanized PTHrP neutralizing antibody is currently in clinical trial for the treatment of breast cancer bone metastasis. Paradoxically, higher PTHrP expression in the primary breast tumor is correlated with a better prognosis and is not associated with the presence of bone metastases[24]. Therefore, the role of PTHrP in bone lesion formation is local, and factor expression may be increased subsequent to the arrival of the metastatic tumor cells in bone.

Other secreted factors also act indirectly on osteoclasts via the RANKL pathway, including vascular endothelial growth factor (VEGF), interleukin-11 (IL-11), and interleukin-6 (IL-6)[2]. Primary breast tumors express the pro-angiogenic factor VEGF and its receptors (VEGFRs)[25-27]. Increased VEGF expression is correlated with increased tumor size and grade[27, 28]. Vascular endothelial growth factor (VEGF) is also highly expressed by breast cancer bone metastases, and VEGFRs are expressed by breast cancer bone metastases, osteoclasts, and osteoclast precursors[27, 29, 30]. VEGF is also a monocyte chemoattractant[27, 30]. VEGF treatment in combination with RANKL, similarly to M-CSF in combination with RANKL, stimulates osteoclast differentiation and bone resorption[27, 29]. Therefore, the high VEGF expression found in breast cancer bone metastases may promote osteoclastic bone resorption and promote lytic bone lesions. Anti-VEGF therapies have been developed for anti-angiogenic therapy, including VEGF antibodies, soluble VEGFRs, VEGFR antibodies, and small-molecule receptor kinase inhibitors[31]. Anti-VEGFR-2 and anti-VEGFR-3 antibody combination therapy decreased lymph node and lung metastases in an orthotopic spontaneous breast cancer metastasis model[32]. Currently, anti-VEGF therapy has only been shown to improve survival in combination with chemotherapy in clinical trials in patients with metastatic colorectal cancer and not in breast cancer[31, 33]. However, since VEGF stimulates osteoclastic bone resorption, anti-VEGF therapy may reduce osteolytic breast cancer bone metastases.

Interleukin-11 (IL-11) also indirectly activates osteoclasts via the RANKL pathway[2]. IL-11 induced bone resorption in calvarial organ culture assays, and this effect was inhibited by Cox inhibitors[34]. IL-11 is expressed by breast cancer cells[17, 35]. It is one of five factors that in combination were identified to contribute to the development of bone metastasis[17]. IL-11 expression was higher in highly bone metastatic MDA-MB-231 subpopulations compared to parental cells[17]. Combined overexpression of IL-11 and osteopontin, but not overexpression of IL-11 alone, increased bone metastases formed by MDA-MB-231 cells[17].

Interleukin-8 (IL-8) is a breast cancer-secreted factor that induces bone resorption in a PTHrP/RANKL-independent manner by acting directly on the IL-8 receptor (CXCR1) on osteoclasts and osteoclast precursors[36, 37]. The chemokine is expressed by breast cancer cell lines, and higher expression is associated with greater osteolytic potential[37]. Patients with breast cancer have elevated IL-8 serum concentrations compared to normal controls, with the highest levels found in patients with advanced disease[38]. MDA-MET breast cancer cells are highly metastatic to bone and differ from parental MDA-MB-231 cells by having increased IL-8 expression and no PTHrP expression, suggesting that IL-8 can drive osteolytic metastases to bone[39]. An IL-8-specific neutralizing antibody inhibited osteoclast formation induced by MDA-MET conditioned media[37]. Combined treatment of mice injected subcutaneously with MDA-MB-231 cells with a human IL-8 antibody and an epidermal growth factor receptor antibody increased overall survival, decreased metastatic spread, and decreased tumor size[40].

5.2 Bone-Forming Breast Cancer-Secreted Factors:

About 15% of breast cancer bone metastases are osteoblastic[6]. Endothelin-1 (ET-1) is a tumor-secreted peptide with a role in osteoblastic bone metastases[16]. ET-1 stimulates osteoblast activity and new bone formation[41]. It is secreted by breast cancers and cell lines that produce osteoblastic and mixed bone lesions in mouse models *e.g.* T47D, MCF-7, ZR75.1, and BT483[16]. Invasive breast tumors express higher ET-1 and ETA receptor than nonneoplastic tissue[42]. Patients with breast cancer and lymph node metastases possess higher ET-1 serum levels than patients without lymph node metastases[42]. Selective inhibition of the endothelin A receptor decreased osteoblastic metastases formed by ET-1-secreting ZR-75-1 breast cancer cells[16]. An ETA receptor antagonist is currently in Phase III clinical trials in men with advanced prostate cancer.

Adrenomedullin (AM) is another secreted peptide that may play a role in osteoblastic breast cancer bone metastases. AM is expressed by human breast cancers and breast cancer cell lines[43]. Higher levels of AM tumor peptide expression and AM plasma levels were found in patients with axillary lymph node metastasis compared to patients without axillary lymph node metastasis[43]. AM stimulates osteoblast proliferation *in vitro* and *in vivo*[44, 45] and induces new bone formation in neonatal mouse calvariae [unpublished data]. Overexpression of AM increased lesion formation in a prostate cancer mouse model, while decreasing AM expression decreased bone lesion formation in a lung cancer bone metastasis mouse model [unpublished data]. Small molecule inhibitors of AM have been developed[46]

that inhibit AM-induced new bone formation in neonatal mouse calvariae [unpublished data]. Such agents may reduce breast cancer bone metastases. Like ET-1, AM is a potent stimulator of pain[47]. Both peptides may contribute to bone metastasis-associated bone pain, which is a major complication of skeletal metastases.

Tumor-secreted platelet derived growth factor-BB (PDGF-BB) may contribute to osteoblastic metastases. PDGFs are multifunctional cytokines that stimulate both osteoclasts and osteoblasts[48]. Breast cancer cells secrete PDGFs and express the PDGF receptor[48, 49]. High PDGF plasma and tumor tissue levels are associated with a poorer prognosis for breast cancer, including increased metastases, lower chemotherapeutic response, and lower survival[50, 51]. Reduction of PDGF-BB in MCF-7 breast cancer cells that overexpress the Neu oncogene decreased osteoblastic bone metastases in nude mice[48]. Overexpression of PDGF-BB in MDA-MB-231 breast cancer cells, which normally produce osteolytic lesions, produced osteoblastic lesions[48]. Gleevac, a selective inhibitor of PDGF receptor tyrosine kinase activity, decreased growth of breast cancer cells injected into the tibia of mice[49]. Such inhibitors could reduce osteoblastic breast cancer bone metastases.

The pro-angiogenic factor connective tissue growth factor (CTGF) is a member of the cysteine-rich CCN protein family and is another breast cancer-secreted factor that stimulates new bone formation[52]. Recombinant CTGF increases bone formation and angiogenesis when injected into the femoral marrow cavity of rats[52]. CTGF is expressed by breast cancer cells[17, 53]. Lower levels of CTGF were detected in breast tumors compared to normal tissues[54]. Low CTGF levels are associated with a poor prognosis, metastasis, local recurrence, and mortality[54]. However, CTGF expression at sites of bone metastases has not been reported. CTGF is a member of the bone metastatic gene profile identified by Kang *et al.* in 2003[17]. Overexpressing CTGF alone did not increase bone metastases formed by MDA-MB-231 in mice[17]. However, overexpressing IL-11, osteopontin, and CTGF together significantly increased the rate and incidence of bone metastases[17]. CTGF neutralizing antibodies decreased osteolytic lesions formed by MDA-MB-231 cells in mice[55]. Thus, CTGF appears to play an important role in bone metastases. The bone microenvironment may induce an increase in CTGF expression.

Another member of the CCN family that stimulates osteoblasts, cysteine-rich protein 61 (Cyr61), may also play a role in bone metastases[56, 57]. Breast cancer tumor tissues expressed higher Cyr61 levels than normal breast tissues[54]. High Cyr61 levels were associated with poor prognosis, nodal involvement, and metastatic disease in breast cancer patients[54]. It was

recently found that a bone-metastatic variant of MDA-MB-231 cells showed increased expression of Cyr61, CTGF, and ET-1, as well as the osteolytic factors IL-11 and IL-8[58].

PTHrP also may play a role in osteoblastic metastases[59]. PTHrP expression is commonly found in prostate cancer cells that produce osteoblastic metastases[60]. PTHrP can be cleaved at residue 23 by the serine proteinase prostate-specific antigen (PSA)[59] that is commonly expressed by breast cancers[61, 62]. The resulting PTHrP fragment does not activate the PTH/PTHrP receptor. PTHrP fragments 1-16 and 1-23 stimulate new bone formation in *ex vivo* calvarial organ cultures, and this stimulation was blocked by an ETAR antagonist, ABT-627, suggesting that PTHrP fragments may stimulate new bone formation through the endothelin A receptor[59]. However, Langlois et al (2005) were unable to show binding of PTHrP 1-16 and 1-23 to the ETA or ETB receptor[60]. Proteolysis may convert PTHrP from an osteolytic to an osteoblastic factor. Therefore, neutralizing PTHrP may also be beneficial for osteoblastic bone metastases, while ETA receptor antagonists may be effective against tumors that make PTHrP fragments but are ET-1-negative.

The bone morphogenetic proteins (BMPs) are a family of growth factors that stimulate bone formation and are part of the TGFβ superfamily[63]. Breast cancer cells express BMPs and BMP receptors[64]. Different BMPs may have both growth inhibitory and stimulatory effects on breast cancer cells[65-66]. Increased expression of the bone morphogenetic protein receptor IB is associated with increased tumor grade, proliferation, cytogenetic instability, and poor prognosis of estrogen receptor-positive breast carcinomas[67]. Overexpression of BMP-2 in MCF-7 breast cancer cells increased the invasive ability of these cells *in vitro* and enhanced estrogen-independent growth in a xenograft mouse model[68]. Overexpression of the BMP antagonist, noggin, in PC3 and LAPC-9 prostate cancer cells decreased osteolytic and osteoblastic lesions, respectively, produced by the prostate cancer cells after injection into the tibia of SCID mice[69, 70].

5.3 Secreted Factors that Can Oppose Bone Metastasis Formation:

Breast cancer cells can secrete factors that oppose bone metastasis formation[14]. These factors are often decreased in breast cancer cells or increased as a host anti-tumor defense response. Increasing these factors in breast cancer patients might be another means to treat breast cancer bone metastases. Osteoprotegerin (OPG) is a secreted decoy receptor for RANKL[71]. OPG is expressed by breast cancer cells, osteoblasts, and bone

stromal cells[72]. Binding of OPG to RANKL prevents RANKL from binding to its receptor RANK on osteoclast precursor cells and osteoclasts, preventing the formation and activation of osteoclasts[71]. Therefore, OPG is a potent inhibitor of osteoclast formation and bone resorption. Breast cancer cells may reduce OPG and increase RANKL expression in the bone to increase osteolysis[72]. Inhibiting RANKL signaling with OPG may inhibit the actions of multiple bone-resorbing tumor factors (*e.g.* PTHrP, IL-11, and VEGF) that induce osteolysis through the RANKL pathway and therefore may be more effective than inhibiting one of these factors alone. Recombinant OPG treatment reduced osteolytic lesion formation, skeletal tumor burden, and tumor-associated osteoclasts formed by MDA-MB-231 breast cancer cells after intracardiac injection in nude mice[73]. A recombinant OPG construct (AMGN-0007) decreased bone resorption without significant adverse effects in a phase I trial using twenty-six patients with breast carcinoma and established lytic bone lesions[74]. However, OPG constructs have not succeeded through clinical trials so far. Small molecule stimulators of OPG expression have also been developed[75]. The small molecule OPG stimulator (Cmpd 5) decreased lytic bone lesions formed by 13762 rat mammary carcinoma cells after intracardiac injection of Fischer-344 rats[75]. However, overexpressing OPG in breast cancer cells increased tumor growth in the tibiae of mice[71], contraindicating the use of small molecule OPG stimulators. Anti-RANKL antibodies have been more successful. The humanized anti-RANKL antibody, denosumab, reduced bone resorption and was well tolerated in patients with multiple myeloma and breast cancer bone metastases[76].

Interleukin-18 (IL-18) enhances the anti-tumor immune response and inhibits osteoclast formation and bone resorption via a mechanism involving granulocyte/macrophage colony-stimulating factor[77-79]. IL-18 upregulates OPG expression by osteoblastic and stromal cells[80]. Patients with breast cancer have higher serum IL-18 levels than patients without breast cancer[78]. Higher IL-18 levels were also found in metastatic patients compared to nonmetastatic with the highest levels found in patients with bone metastasis[78, 81]. IL-18 injections into nude mice reduced osteolytic bone metastases formed by intracardiac injection of MDA-MB-231 breast cancer cells but had no effect on subcutaneous tumor growth[82]. Systemic administration of recombinant IL-18 in humans could reduce breast cancer bone metastases.

Soluble frizzled related protein 1 (Sfrp1) is a breast cancer secreted protein that inhibits the Wnt signaling pathway[83]. The Wnt signaling pathway has a known role in osteogenesis and oncogenesis[84]. Wnt signaling activates osteoblasts and Wnt signaling inhibitors like Sfrp1 and dickkopf-1

(DKK-1) inhibit this activation[84]. Activation of the Wnt signaling pathway also promotes mammary carcinogenesis[83, 85]. Downregulation of repressors of Wnt signaling, Sfrp1 and the transcription factor TCF-4, was identified in a subset of breast cancers[83]. Deletion of the chromosomal region containing Sfrp1 is often detected in breast cancer[86]. Aberrant hypermethylation (gene-silencing) of Sfrp1 was also associated with an unfavorable prognosis for breast cancer[86]. Increasing Wnt activity by knocking down DKK-1 expression with DKK-1 short hairpin RNA caused osteolytic PC3 prostate cancer cells to induce osteoblast activity[87]. Decreasing Wnt activity by overexpressing DKK-1 converts prostate cancer cells with a mixed osteolytic/osteoblastic phenotype to an osteolytic phenotype[87]. Wnt signaling contributes to prostate cancer osteoblastic bone metastasis formation[87] and may in the same way contribute to breast cancer bone metastasis. Suppression of the Wnt signaling pathway may reduce osteoblastic bone metastasis. A green tea compound (-)-Epigallocatechin 3-gallate (EGCG) inhibits Wnt signaling and reduces breast cancer cell proliferation and invasiveness[88]. Green tea consumption has been correlated with reduced recurrence of breast cancers in Japanese women[88]. Oral administration of EGCG reduced breast cancer tumor progression in animal models[88]. EGCG may reduce osteoblastic bone metastases. However, Wnt signaling inhibition has also been suggested to be one of the mechanisms that multiple myeloma induces bone destruction by inhibiting bone formation[89, 90]. Multiple myeloma cells and multiple myeloma patients with advanced osteolytic lesions secreted the Wnt inhibitor, secreted frizzled-related protein-2 (Sfrp-2) and Sfrp-2 inhibits bone formation[89]. Further research is needed to test the role of the Wnt signaling inhibitors in breast cancer bone metastasis.

6. CURRENT PROBLEMS AND POSSIBLE FUTURE TREATMENT DIRECTIONS: IDENTIFYING UPSTREAM REGULATORS TO TARGET MULTIPLE FACTORS INVOLVED IN BREAST CANCER BONE METASTASIS

The approved treatment for breast cancer bone metastases is antiresorptive bisphosphonates[2]. Bisphosphonates bind to bone matrix and reduce osteoclastic bone resorption[14]. They promote osteoclast apoptosis, while their effects *in vivo* on osteoblasts and tumor growth remain controversial[14]. Bisphosphonates reduce bone pain and skeletal fractures but do not improve overall survival[2]. Additional classes of antiresorptive agents

include anti-RANKL antibodies and cathepsin K inhibitors. These are in clinical development but are not yet approved for patient use. Anti-RANKL antibodies prevent interaction of RANKL with RANK, interfering with formation and activation of osteoclasts[76]. Cathepsin K inhibitors inhibit one of the proteolytic enzymes secreted by osteoclasts, cathepsin K, that is necessary for bone resorption[91]. Out of the three groups, Cathepsin K inhibitors are the only agents that do not prevent osteoclast formation or induce osteoclast death. If osteoclasts have other functions in bone beyond osteolysis, drugs that allow osteoclast formation, but block their bone resorptive activity, may have fewer side effects.

Current treatment flaws leave the need for the development of more effective therapies. This chapter has demonstrated a method of targeting tumor-secreted factors such as PTHrP to treat breast cancer bone metastases. Many additional factors are involved in breast cancer bone metastases. The important question is: How to find the best target(s) out of the long list of factors to effectively cure breast cancer bone metastases? The best strategy may be to target multiple tumor-secreted factors. Kang *et al.* (2003) demonstrated that not one, but a combination of four to five factors were necessary for bone metastasis formation[17]. They identified a bone metastatic gene profile consisting of forty-three genes with varying functions, among which included the bone-resorbing factor IL-11 and the bone-forming, angiogenic factor CTGF[17]. These genes only in combination enhanced bone metastasis formation produced by poorly metastatic MDA-MB-231 cells[17]. Therefore, multiple factors are important in bone metastasis formation and targeting multiple factors may be more effective in treating breast cancer bone metastases than targeting one factor alone. Indeed, breast cancers secrete multiple factors from the lists of both bone-resorbing and bone-forming proteins[6]. Therefore, a more effective treatment may be to target an upstream regulator of multiple factors. Many of the known tumor-secreted factors, both osteolytic and osteoblastic, are regulated by the hypoxia-induced Hif-1α pathway and the TGFβ signaling pathway[8, 17, 92]. Both pathways are active in the bone microenvironment and are important targets for treatment of bone metastases. TGFβ inhibitors have been effective in blocking bone metastases in preclinical models[9-12]. Additional upstream regulators need to be identified and may prove to be more effective treatment targets for breast cancer bone metastasis treatment. Combining this approach of targeting tumor-secreted factors with other therapies (bisphosphonates and chemotherapeutics) may improve treatment[93]. Inhibitors of tumor-secreted factors may be important adjuvant therapies for breast cancer bone metastasis.

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Supporting Data:

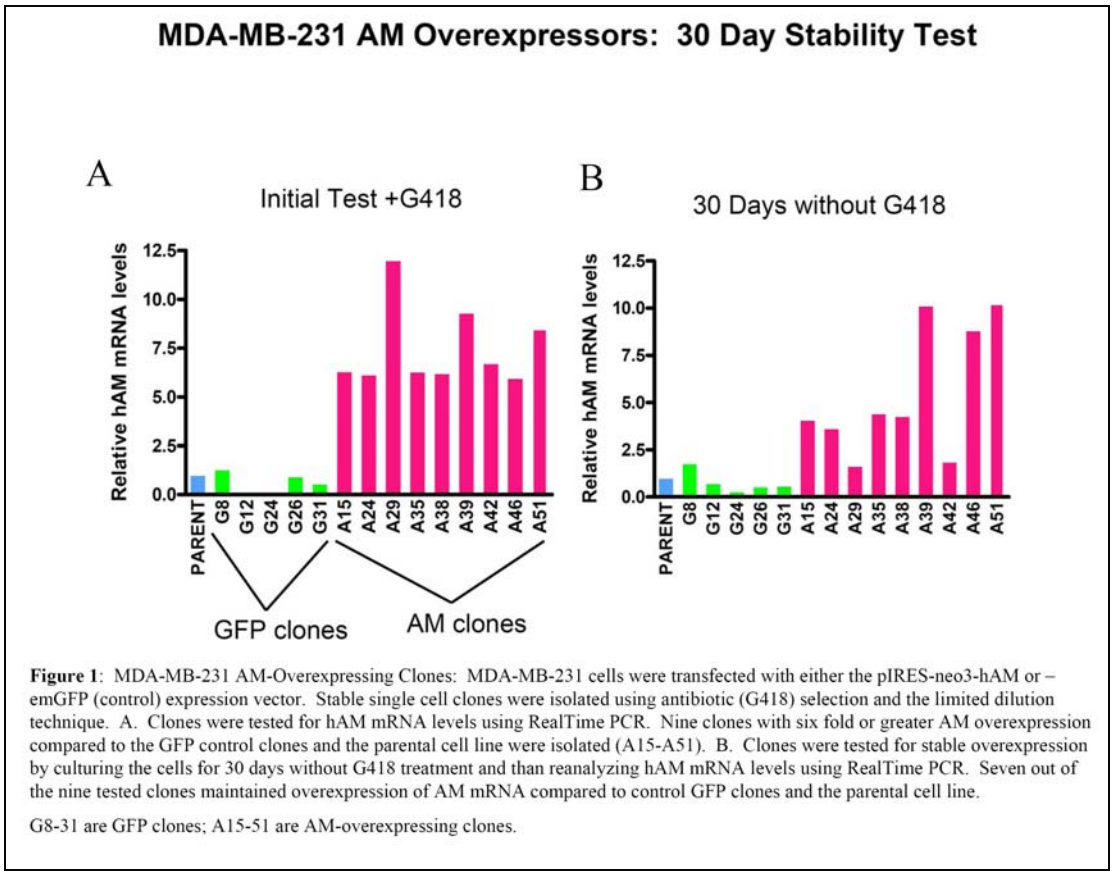


Figure 1: MDA-MB-231 AM-Overexpressing Clones: MDA-MB-231 cells were transfected with either the pIRES-neo3-hAM or –emGFP (control) expression vector. Stable single cell clones were isolated using antibiotic (G418) selection and the limited dilution technique. A. Clones were tested for hAM mRNA levels using RealTime PCR. Nine clones with six fold or greater AM overexpression compared to the GFP control clones and the parental cell line were isolated (A15-A51). B. Clones were tested for stable overexpression by culturing the cells for 30 days without G418 treatment and than reanalyzing hAM mRNA levels using RealTime PCR. Seven out of the nine tested clones maintained overexpression of AM mRNA compared to control GFP clones and the parental cell line.

G8-31 are GFP clones; A15-51 are AM-overexpressing clones.

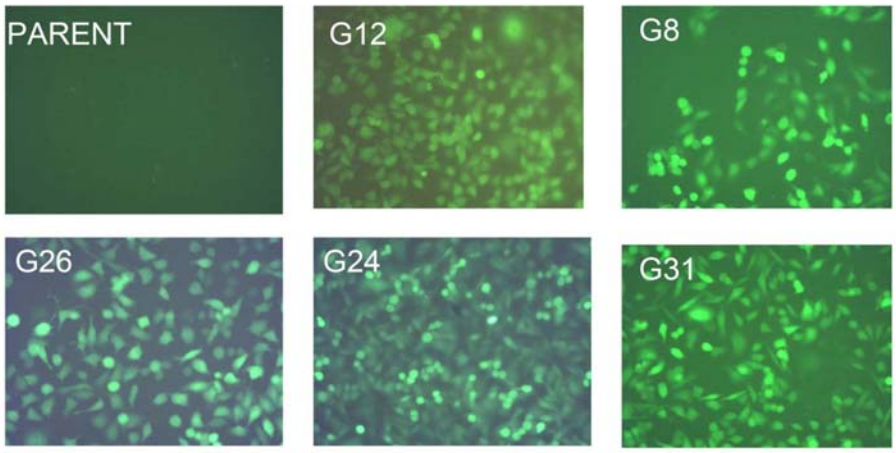


Figure 2: GFP-expressing MDA-MB-231 clones: MDA-MB-231 GFP-clones were grown for thirty days without G418 treatment to test for stable expression of the green fluorescent protein. Cells were tested for stable expression of GFP by detecting green fluorescence using a fluorescent microscope. Five clones maintained detectable expression of GFP. PARENT=parental MDA-MB-231 cell line; G12, G8, G26, G24, and G31=different GFP-expressing MDA-MB-231 clones

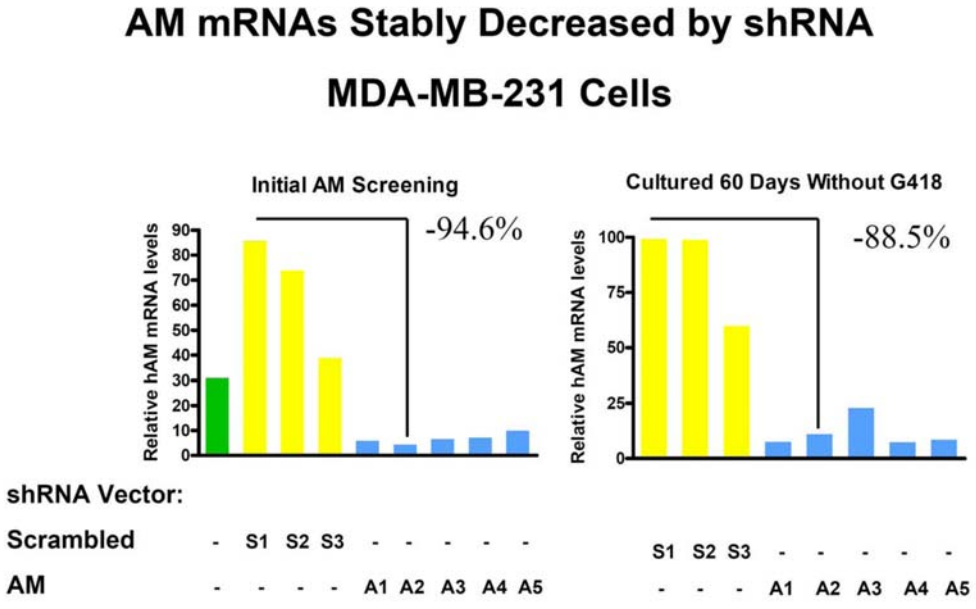
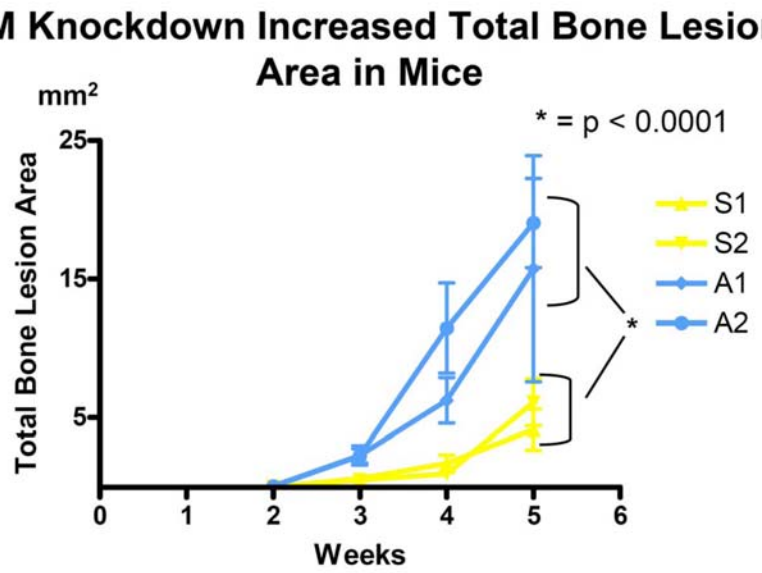


Figure 3: MDA-MB-231 AM Knockdown Cells: A. MDA-MB-231 cells were transfected with either a shRNA targeting the hAM gene or a scrambled version of that sequence as a control. Stable single cell clones were isolated using antibiotic (G418) selection and the limited dilution technique. Clones were tested for hAM mRNA expression using RealTime PCR. Five clones were identified with about a 90% AM mRNA knockdown compared to the control clones. B. Clones were tested for stability of the knockdown by culturing the cells without antibiotic selection (G418) for 60 days. Clones were than retested for expression of hAM using RealTime PCR. Clones maintained AM mRNA knockdown after 60 days without G418 treatment.



•7/7 (100%) mice injected with S1 & 4/4 (100%) with S2 developed lesions

•7/9 (77.8%) mice injected with A1 & 9/11 (88.8%) with A2 developed lesions

Figure 4: MDA-MB-231 AM Knockdown Bone Metastasis Experiment: Two AM knockdown (A1 and A2) and two scrambled control (S1 and S2) MDA-MB-231 clones were injected into the left cardiac ventricle of nude mice (n=12 mice/group) to produce a mouse model of bone metastasis. Mice were x-rayed over 5 weeks and than the Metamorph imaging software was used to quantitate total bone lesion area per mouse from the x-rays. AM knockdown clones caused greater osteolytic lesion area (17.9 vs. 4.8 mm2 at 5 wks; $p<0.0001$) than scrambled control cells.

S1 & S2= scrambled control clones; A1 & A2= AM knockdown clones

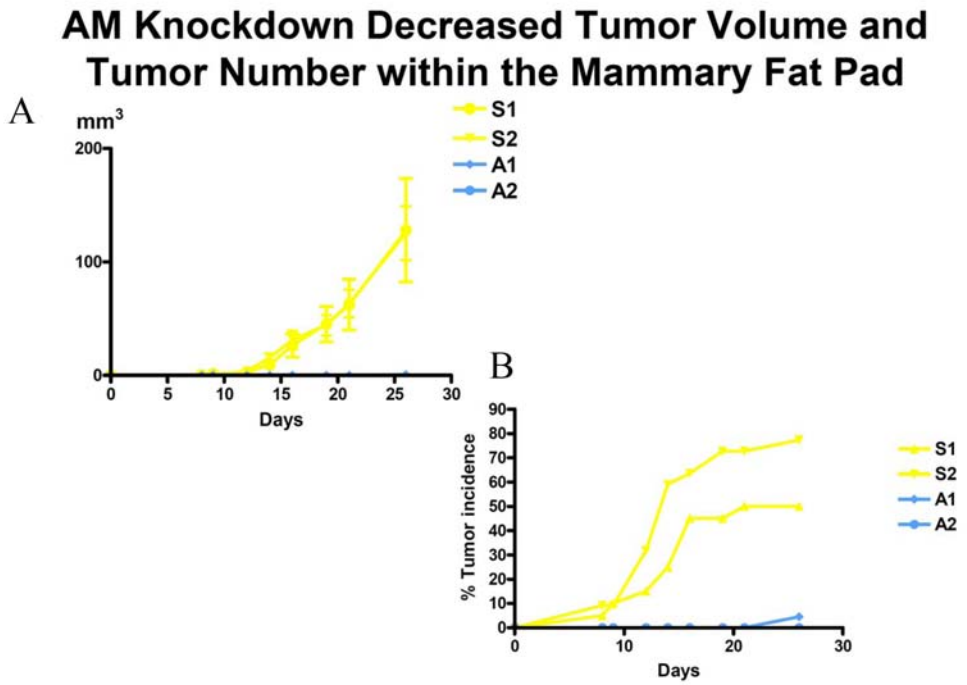


Figure 5: MDA-MB-231 AM Knockdown Mammary Fat Pad Experiment: Two scrambled control (S1 & S2) and two AM knockdown (A1 & A2) MDA-MB-231 clones were injected into the mammary fat pad of female nude mice (n=10mice/group). Tumor length and width were measured using a caliber. A. AM knockdown clones exhibited decreased tumor growth (0.5mm3 vs. 126.4mm3 on day 26) in the mammary fat pad compared to the control clones. B. AM knockdown clones exhibited decreased tumor take (2.5% vs. 64.3%; $p<0.0001$) in the mammary fat pad compared to the control clones.

S1 & S2= scrambled control clones; A1 & A2 = AM knockdown clones

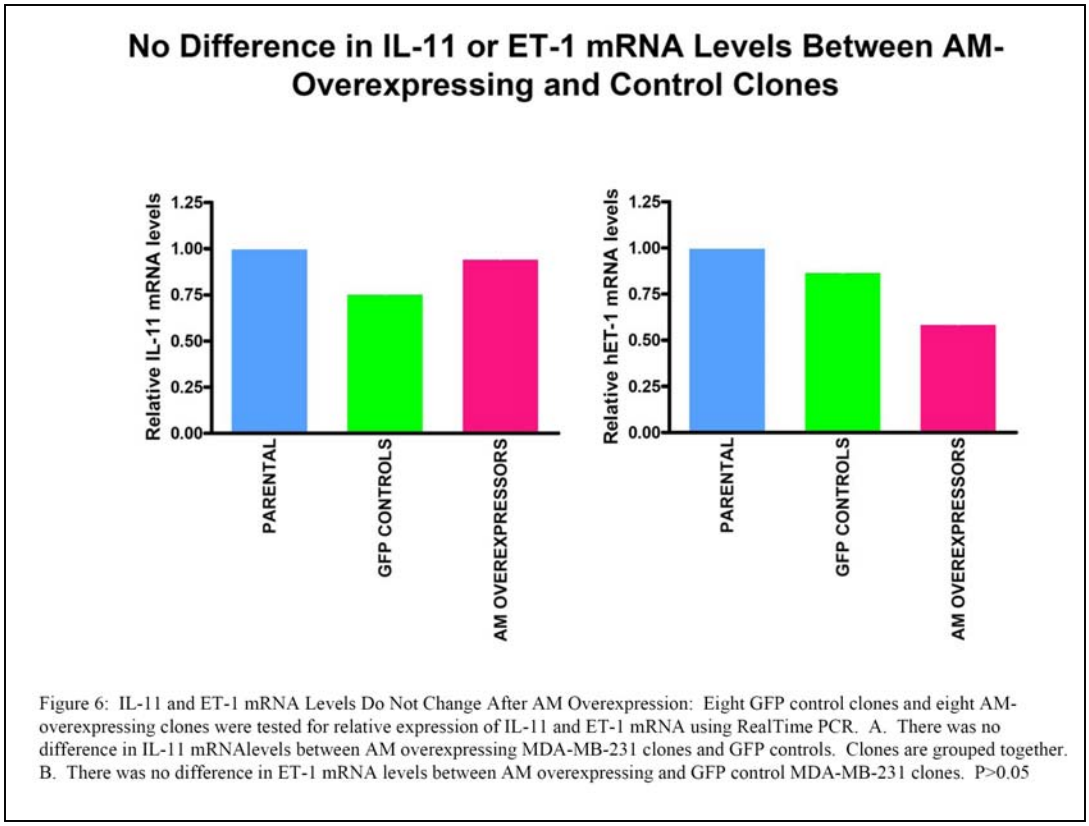


Figure 6: IL-11 and ET-1 mRNA Levels Do Not Change After AM Overexpression: Eight GFP control clones and eight AM-overexpressing clones were tested for relative expression of IL-11 and ET-1 mRNA using RealTime PCR. A. There was no difference in IL-11 mRNAlevels between AM overexpressing MDA-MB-231 clones and GFP controls. Clones are grouped together. B. There was no difference in ET-1 mRNA levels between AM overexpressing and GFP control MDA-MB-231 clones. $P>0.05$